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Control of *Vibrio alginolyticus* in *Artemia* culture by treatment with bacterial probiotics

L. Villamil, A. Figueras, M. Planas, B. Novoa*

Instituto de Investigaciones Marinas, Consejo Superior de Investigaciones Cientificas (CSIC), Eduardo Cabello, 6 Marinas, 36208 Vigo, Spain

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Abstract

In order to determine the influence of six lactic acid bacterial (LAB) strains, lactic acid and major extracellular products (ECPs) of LAB on the bacterial load of *Artemia* culture, two experiments were carried out. In both experiments, the dominant bacterial species were identified as *Vibrio alginolyticus*. The treatment with *Lactobacillus brevis* (1×10^8 bacteria/ml) and lactic acid (0.1 g/l) reduced the load of this bacterium in the *Artemia* culture water.

Biochemical and morphological characteristics of the *V. alginolyticus* strains isolated from *Artemia* culture were determined. For comparative purposes, another *V. alginolyticus* strain isolated from diseased turbot larvae was also included.

The in vitro antibacterial activity of extracellular products (ECPs) from LAB, as well as lactic acid (0.1 and 0.05 g/l), was assessed against both *V. alginolyticus* strains. ECPs from LAB were able to inhibit the growth of *V. alginolyticus*, especially those from *L. brevis*. In the case of lactic acid treatments, no significant inhibition of *V. alginolyticus* growth was observed.

Keywords: *Vibrio alginolyticus*; Lactic acid bacteria; Bacterial probiotics; Lactic acid; Antibacterial activity

1. Introduction

The incidence of microbial diseases has increased dramatically in accordance with the growth of fish larvae production (Toranzo et al., 1994). In the case of turbot

* Corresponding author. Tel.: +34-986-214463; fax: +34-986-292762.
E-mail address: virus@iim.csic.es (B. Novoa).

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(*Scophthalmus maximus* L.) culture, high mortalities occur during larval first feeding due to the incidence of pathogenic and opportunistic bacteria (May, 1973; Grisez et al., 1996). Different authors have suggested that this infection happens mainly through the food chain (Sera and Kumata, 1972; Campbell and Buswell, 1983; Muroga et al., 1987) and especially during the feeding with *Artemia* (Chair et al., 1994; Grisez et al., 1996).

One of the most frequently used procedures to avoid the incorporation of undesirable bacteria to the fish culture is the administration of antibiotics in the water (Brown, 1989) or via *Artemia* (Verpraet et al., 1992; Touraki et al., 1999). However, there is a major concern with respect to the use of antibiotics since they could promote the development of antibiotic-resistant bacteria in fish and the environment (Alderman and Hastings, 1998; Brown, 1989; Bjorklund et al., 1991; Smith et al., 1994; Pedersen et al., 1995; Hameed and Balasubramanian, 2000). In order to increase larval survival, several approaches such as improving husbandry nutrition (Reitan et al., 1993; Roennestad et al., 1999), water quality (Vadstein et al., 1993; Skjermo et al., 1997), disinfecting fish eggs (Salvesen and Vadstein, 1995) and UV treatment (Munro et al., 1999) have been proposed. The use of nonspecific immunostimulants (Robertsen et al., 1990; Anderson, 1992; Figueras et al., 1997) and bacterial probiotics such as lactic acid bacteria (Gatesoupe, 1991, 1994, 1999; Ringø and Gatesoupe, 1998; Verschuere et al., 2000) have also been proposed.

Probiotics are defined as microbial dietary adjuvants that beneficially affect the host physiology by modulating the mucosal and systemic immunity, as well as improving microbial balance by preventing the colonization of undesirable bacteria in the intestinal tract (Naidu et al., 1999). The role of lactic acid bacteria (LAB) as probiotics in the digestive tract has been extensively studied in endothermic animals and humans (Aiba et al., 1998; Kontula et al., 1998; Kirjavainen et al., 1999). In fish, LAB were recently described as part of the normal microflora (Strøm and Olfasen, 1990; Ringø and Strøm, 1994; Ringø et al., 1998; Robertson et al., 2000). Exogenous LAB administration has been associated with pathogenic bacterial growth inhibition (Lewus et al., 1991; Gildberg et al., 1995; Santos et al., 1996), fish growth promotion (Noh et al., 1994) and in some cases with an increased survival of experimental infected fish (Gatesoupe, 1994; Gildberg et al., 1997; Robertson et al., 2000). Preventing colonization of detrimental bacteria with selected bacterial strains has been proposed as an important alternative for microbial control during *Artemia* culture (Verschuere et al., 1999). It has also been demonstrated that some of these selected bacterial strains can prevent the growth of bacterial pathogens such as *Vibrio proteolyticus* in the *Artemia* culture (Verschuere et al., 2000).

In the current work, we have studied the effect of six LAB strains and lactic acid in the bacterial load of *Artemia* culture. Since we observed an important reduction of *Vibrio alginolyticus* strains (F 236 and F 237) isolated as dominant bacterium in the *Artemia* culture, further experiments were conducted with these isolates and with another *V. alginolyticus* strain (Hq 221) isolated from diseased turbot larvae. The in vitro antibacterial activity of lactic acid and ECPs from LAB against both bacteria was determined. Biochemical and morphological characteristics of all strains were also included.

2. Material and methods

2.1. Lactic acid bacterial strains

Five LAB strains were obtained from the Spanish type culture collection (CECT): *Lactobacillus casei* (CECT 4043), *Lactobacillus brevis* (CECT 815), *Lactobacillus helveticus* (CECT 541), *Lactococcus lactis* spp. *lactis* (CECT 539) and *Leuconostoc mesenteroides* spp. *mesenteroides* (CECT 4046). *Pediococcus acidilactici* (NRRL B-5627) was obtained from the Northern Regional Research Laboratory, USA. All the LAB strains were grown overnight at 30 °C in Man Rogosa and Sharpe broth (MRS), washed and resuspended in sterile phosphate saline buffer (PBS). These LAB species were chosen since they are known to be safe for human use. Furthermore, they were referenced in other investigations as bacterial probiotics in aquaculture or as bacteria with high antimicrobial activity (SchrØde et al., 1980; Shiri Harzevili et al., 1998; Gatesoupe, 1991, 1994, 2002; Nikoskelainen et al., 2001).

2.2. Artemia culture

Artemia cysts (INVE) were hatched in filtered seawater at 28–30 °C under continuous aeration and light intensity. After 24 h of incubation, *Artemia* nauplii (instar I) hatched out and were used for experimental studies.

2.3. LAB and lactic acid administration to Artemia culture

Six LAB strains were separately administered to *Artemia* nauplii at a final concentration of 1×10^8 bacteria/ml (Vázquez, 2001). In another series of experiments, lactic acid (Sigma) at different final concentrations (0.005, 0.01, 0.05, 0.1 g/l) were administered to *Artemia* nauplii. In both experiments, *Artemia* culture was maintained for 24 h with aeration at 15 °C. After this time, samples of 5 ml were aseptically taken to determine the bacterial load, as described below.

2.4. Sample processing

To separate *Artemia* from culture water, samples from each treatment were passed over a sterile 100-μm mesh. The *Artemia* trapped in the filter were rinsed with 10 ml of sterile seawater (ssw), resuspended in 1 ml of ssw and aseptically homogenized. Samples (1 ml) from the culture water were aseptically stored in sterile tubes until used.

In order to determine the bacterial load in *Artemia* and culture water, samples were serially diluted and 100 μl of each dilution was plated on marine agar (MA) (Panreac Quimica) and on thiosulphate citrate bile sucrose (TCBS) (Panreac Quimica). Plates were then incubated for 48–72 h at room temperature (22 °C). After this time, the colonies were counted and the amount of bacteria was calculated as colony forming unit (CFU) per ml. Dominant colony types on plates were visually selected on the basis of different colony appearance and abundance. The selected colonies from each plate were isolated and re-streaked on fresh MA to ensure purity. For long-term preservation,

cultures were frozen at −80 °C in tryptone soy broth containing 1% NaCl (TSB-1) and 15% glycerol (v/v).

2.5. Characterization of bacterial isolates

Pure cultures of the bacterial strains were subjected to standard morphological, physiological and biochemical plate and tube tests. Since we focus this study in the *Vibrio* genus, gram character (Buck, 1982), oxidase test, morphology, motility, sensitivity against O/129 and growth on TCBS were the tests used to identify the isolates that are members of this genus. Identification of *V. alginolyticus* species was achieved following the scheme of Alsina and Blanch (1994) and Bergey's Manual of Systematic Bacteriology (1984, vol. 1, section 5). In parallel, the commercial miniaturized Api 20-E (Bio Mérieux) was also used.

Drug resistance patterns of the *V. alginolyticus* isolates were determined by disc diffusion method on Mueller–Hinton agar (Oxoid) supplemented with 1% NaCl. The chemotherapeutic agents and their concentrations (μg/disc) were ampicillin (10), chloramphenicol (30), furazolidone (300), nitrofurantoin (300), oxolinic acid (2), oxytetracycline (30), streptomycin (10), tetracycline (30) and trimethoprim-sulphamethoxazole (25).

The enzymatic activity of the *V. alginolyticus* isolates was determined with the Api ZYM system (Bio Mérieux) according to the manufacturer's instructions. For comparative purposes, *V. alginolyticus* (Hq 221) isolated from diseased turbot larvae was included in all the characterization tests.

2.6. Antibacterial activity of extracellular products from LAB and lactic acid

We determined the in vitro capacity of extracellular products (ECPs) from the six LAB species and lactic acid to inhibit the growth of *V. alginolyticus* strains isolated from *Artemia* culture and from diseased turbot larvae.

2.6.1. Extraction of LAB ECPs

The extraction of ECPs was performed according to Cabo et al. (1999). Briefly, all the LAB strains were grown overnight at 30 °C in MRS broth. After incubation, the pH was adjusted to 3.5 with 2 N CIH, then heated at 80 °C for 3 min and centrifuged at $500 \times g$ for 30 min. The supernatant containing the LAB ECPs was filtered through a 0.45-μm filter, buffered at pH 6.0 and stored in aliquots at −80 °C.

2.6.2. Bactericidal assay

Bacterial strains to be tested were grown overnight at room temperature (22 °C) in TSB-1. The assay was performed in triplicate in 96-well plate by dispensing 50 μl of the bacterial suspension (10^8 bacteria/ml) per well and 50 μl of the corresponding treatment: ECPs (undiluted or previously diluted to one half) or lactic acid (0.05 and 0.1 g/l). Controls were included by incubating the bacteria with MRS instead of ECPs, or with PBS at pH 3.5 instead of lactic acid. After 15 h of incubation, changes in optical density (OD)

(600 nm) were measured and the percentage of bacterial survival was calculated as follows:

$$\text{OD treated wells}/\text{OD untreated wells} \times 100.$$

2.7. Statistics

The data were compared using a Student's *t*-test performed using the computer program Excel 98. Results are expressed as the mean ± standard deviation and the differences were considered significant at $p < 0.05$.

3. Results

3.1. LAB administration to Artemia culture

The majority of isolated bacteria from this experiment belonged to the genus *Vibrio*. They were Gram-negative motile rods sensitive to the vibriostatic agent O/129 and were able to grow in TCBS medium. From all the colonies, one with high capacity to lyse surrounding colonies and to overgrow them was isolated and further identified as *V. alginolyticus* (F 236). In the culture water, a total bacterial average of 10^8 CFU/ml was detected (Fig. 1a), while an average of 10^4 CFU/*Artemia* was recorded (Fig. 1b). The treatment with *L. brevis* (CECT 815) and *L. casei* (CECT 4043) almost completely eliminated *V. alginolyticus* from *Artemia*, whereas in water, it was only eliminated by treatment with *L. brevis*.

3.2. Lactic acid administration to Artemia culture

Again, in this experiment, most of the isolated bacteria belonged to the *Vibrio* genus. The bacterial colony with high capacity to lyse surrounding colonies and to overgrow them was also identified as *V. alginolyticus* (F 237). The total amount of bacteria isolated from culture water and *Artemia* was similar to that reported in the experiment with LAB treatments. The treatment with the highest concentration of lactic acid (0.1 g/l) diminished the load of *V. alginolyticus* in *Artemia* culture. However, this treatment was not able to completely eliminate this bacterium from *Artemia* culture (Fig. 2).

3.3. Characterization of bacterial isolates

The main biochemical characteristics of *V. alginolyticus* strains isolated from *Artemia* culture (F 236 and F 237) and diseased turbot larvae (Hq 221) are included in Table 1. All bacterial strains presented a similar profile: they were nonpigmented colonies in TSA-1 while in TCBS, they formed yellow (y) colonies. These isolates require Na^+ to grow and were positive for oxidase, catalase, indole and Voges–Proskauer. Their

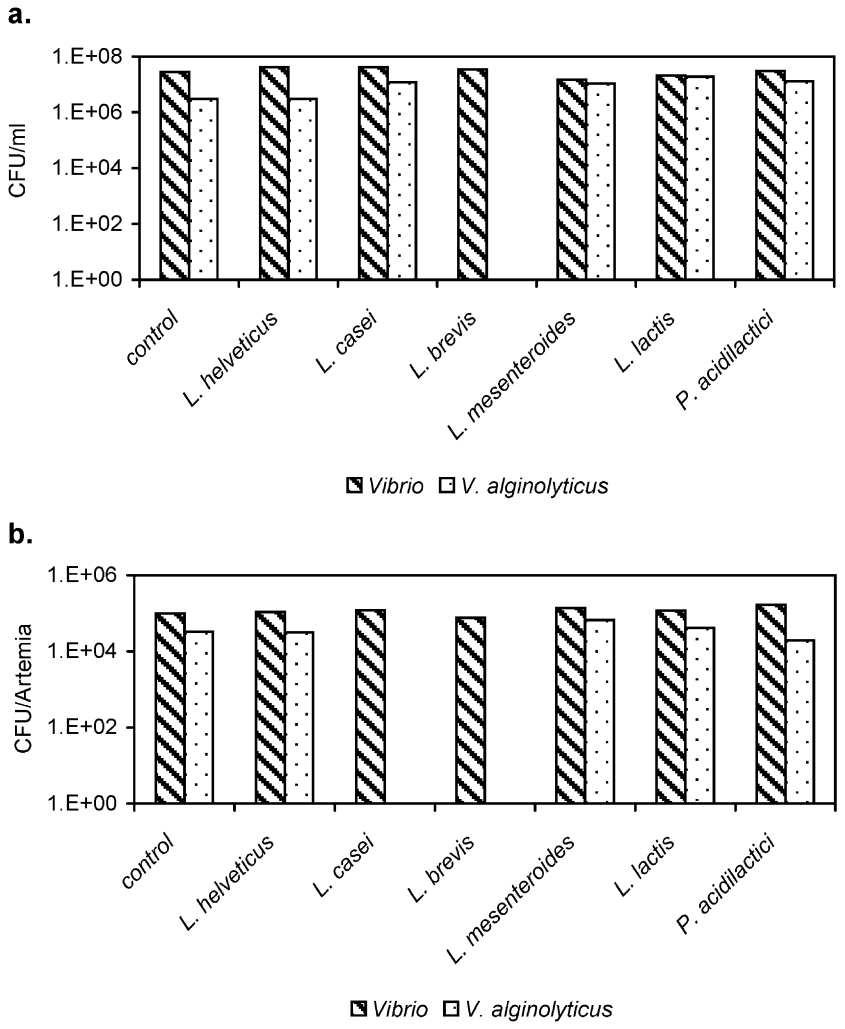

Fig. 1. Effect of administration of six LAB strains (1×10^8 bacteria/ml) in the bacterial load of *Artemia* culture. Dominant bacteria isolated from (a) water and from (b) *Artemia* nauplii. Data were expressed as CFU/ml in the case of water and as CFU/*Artemia* in the case of nauplii.

activities with arginine dihydrolase and lysine decarboxylase were negative; however, they differ on ornithine decarboxylase. The strains isolated from *Artemia* culture were positive and the one isolated from the diseased turbot larvae was negative. All isolates presented similar sensitivity patterns with tested antibiotics, chloramphenicol, tetracycline and oxytetracycline being the most effective, while both isolates were resistant to ampicillin (Table 1).

The highest enzymatic activities observed in both *V. alginolyticus* strains were alkaline phophatase, leucine arylamidase, acid phosphatase and naphthol-AS-bi-phosphohydrolase.

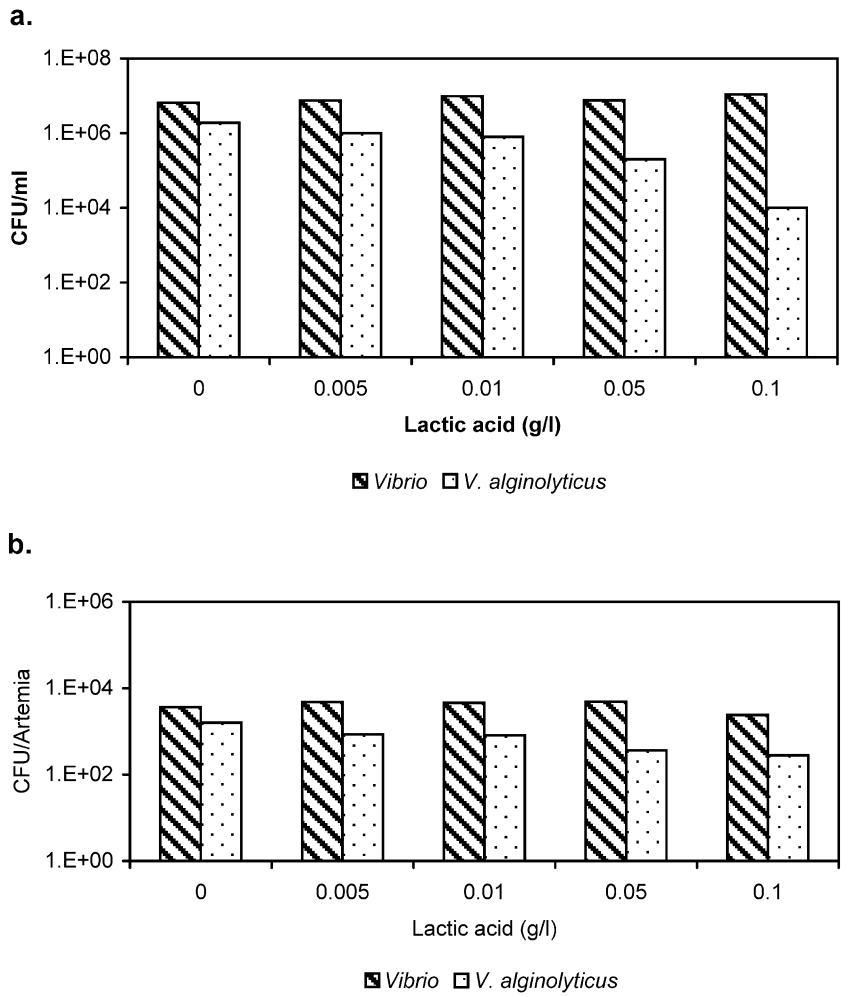

Fig. 2. Effect of administration of lactic acid at different final concentrations (0.005, 0.01, 0.05 and 0.1 g/l) on the bacterial load of *Artemia* culture. Dominant bacteria isolated from (a) water and from (b) *Artemia* nauplii. Data were expressed as CFU/ml in the case of water and as CFU/*Artemia* in the case of nauplii.

The bacterial strains isolated from *Artemia* culture (F 236 and F 237) exhibited a strong activity of α-chymotrypsin, whereas the strain isolated from diseased turbot larvae (Hq 221) showed a weak reaction.

3.4. Bactericidal assay

V. alginolyticus isolated from *Artemia* culture and diseased turbot larvae were sensitive to ECPs from some LAB strains. ECPs from *L. brevis* caused the strongest

Table 1
Main phenotypic characteristics of the strains of *V. alginolyticus* isolated from diseased turbot larvae (Hq 221) and from *Artemia* culture (F 236–F 237)

Characteristics	*V. alginolyticus* (F 236 and F 337)	*V. alginolyticus* (Hq 221)
Gram	–	–
Motility	+	+
Oxidase	+	+
Catalase	+	+
Pigment	–	–
TCBS	+ (y)	+ (y)
Swarming on solid media	+	+
Arginine dihydrolase	–	–
Ornithine decarboxylase	+	–
Lysine decarboxylase	+	+
O/F glucose	+/+	+/+
Citrate	+	+
Indole	+	+
Voges–Proskauer	+	+
ONPG	–	–
H_2S production	–	–
Na^+ required for growth	+	+
Gelatinase	+	+
Urease	–	–
Growth at (C):		
4	–	–
15	+	+
37	+	+
Acid from:		
Glucose	+	+
Mannitol	+	+
Inositol	–	–
Sorbitol	–	–
Rhamnose	–	–
Sucrose	+	+
Melibiose	–	–
Amygdalin	–	–
Resistance/sensitivity to:		
O/129	S	S
Ampicillin	R	R
Chloramphenicol	S	S
Nitrofurantoin	S	S
Oxolinic acid	S	S
Oxytetracycline	S	S
Streptomycin	S	S
Tetracycline	S	S
Trimethoprim-sulphametoxazole	S	S

R: resistant; S: sensitive.

inhibition of both *V. alginolyticus* strains, even if diluted to one half. However, diluted ECPs from other LAB were not able to cause a significant reduction in the growth of *V. alginolyticus* (Fig. 3).

In the case of lactic acid, a slight but not significant inhibition of *V. alginolyticus* growth was observed with the highest dose used (0.1 g/l) (data not shown). Higher lactic acid concentrations were not assessed since they were found to be toxic for *Artemia* culture.

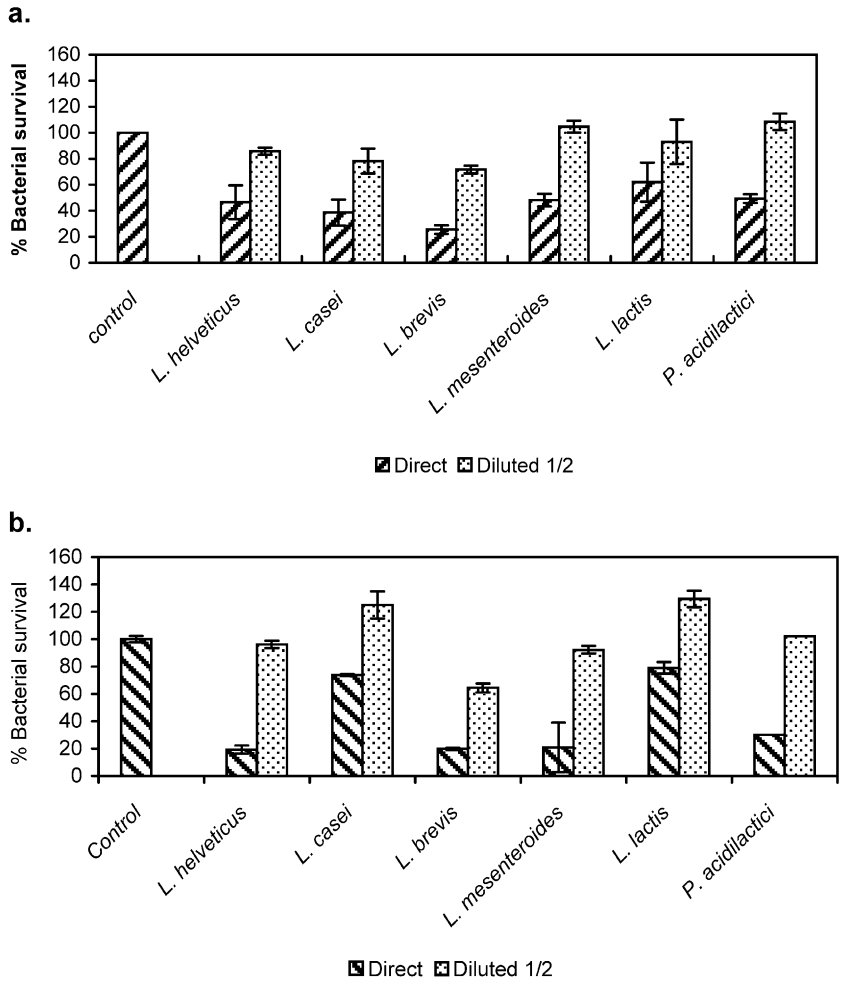

Fig. 3. Growth inhibition of *V. alginolyticus* isolated from (a) *Artemia* culture and from (b) diseased turbot larvae by incubation with ECPs (direct or previously diluted to 1/2) from LAB for 15 h. Data were presented as mean percentage of bacterial survival ± SD.

4. Discussion

Bacteria from the *Vibrio* genus are most frequently associated with mortalities in marine fish farms (Horne et al., 1977; Devesa et al., 1985; Toranzo et al., 1993). *V. alginolyticus* has been described as a pathogenic bacteria for turbot (Austin et al., 1993), sea bream (*Sparus aurata* L.), (Colorni et al., 1981; Balebona et al., 1998) and grouper (*Epinephelus malabaricus*) (Lee, 1995). It has also been reported as opportunistic bacteria for sea mullet (*Mugil cephalus* L.) (Burke and Rodgers, 1981) and red sea bream (*Pagrus major*) (Muroga et al., 1987), while other studies described this bacteria as common intestinal microflora in several marine fish species (Grisez et al., 1997).

Manipulation of bacterial load present in rotifers and *Artemia* for fish feeding may constitute a valuable mechanism to increase larval growth and survival rates (Noh et al., 1994; Gatesoupe, 1994; Robertson et al., 2000). Results obtained in the present study suggest that treatments with *L. brevis* and *L. casei* significantly diminish *V. alginolyticus* load in *Artemia* culture. In the case of lactic acid, only the highest dose used was able to reduce bacterial numbers mainly in the culture water. During the metabolism, LAB produce a range of ECPs that could inhibit bacterial growth such as lactic acid (Lindgren and Clevström, 1978; Alakomi et al., 2000), organic acids (Midolo et al., 1995), hydrogen peroxide, carbon dioxide (Naidu et al., 1999) and bacteriocins. Bacteriocins are bactericidal or bacteriostatic polypeptides that are mostly active against closely related bacteria (Klaenhammer, 1998) and gram-positive microbes (Galvin et al., 1999; Hurst, 1981; Severina et al., 1998). However, some LAB also seem to be antagonistic against gram-negative bacteria such as *Vibrio anguillarum*, *Vibrio salmonicida* and *Proteus vulgaris* (Strøm, 1998 in Ringø and Gatesoupe, 1988). The carnocin from *Carnobacterium piscicola* was also found to be effective against *Aeromonas hydrophila* (Lewus et al., 1991).

In vitro bactericidal activity results were well correlated with in vivo observations. Experiments showed that lactic acid slightly, but not significantly, inhibited *V. alginolyticus* growth; however, a major growth inhibition was obtained when the bacteria were incubated with ECPs from LAB. These results indicate that the bacterial inhibition may be caused by the synergism of various compounds produced as ECPs of LAB and not by lactic acid alone. Undissociated weak acids like lactic acid possess the ability to cross membranes of microorganisms, become dissociated inside and acidify the interior causing the expulsion of H^+ ions from the cells; this action weakens the cells and makes them more susceptible to bacteriocins and other bactericidal compounds (Nykänen et al., 1998). Other mechanisms that could inhibit the growth of undesirable bacteria such as competition for nutrients, available energy or adhesion sites (Gatesoupe, 1999; Verschuere et al., 2000) should be considered. LAB have also been successfully applied in the rotifer (*Brachionus plicatilis*) culture. Gatesoupe (1991) reported that a commercial preparation of live *Lactobacillus plantarum* decreased the proportion of *Aeromonas salmonicida*-like bacteria associated with rotifers, and Harzevili et al. (1988) demonstrated that *L. lactis* (AR21) exhibited an inhibitory effect against *V. anguillarum* in rotifer culture at suboptimal conditions.

Treatment of *Artemia* with acids such as hypochlorite has been demonstrated to completely disinfect cysts. However, these are rapidly recolonized with bacteria (Sogerloos et al., 2001). In order to maintain the inhibition of undesirable bacteria, it is possible

that LAB, as well as chemical compounds, have to be supplied on a regular basis. Jöborn et al. (1997) reported that the addition of high doses of LAB caused a temporary change in the composition of fish intestinal microbial communities, but within a few days after the intake had stopped, the exogenous added bacteria showed decrease in numbers and were lost from the gastrointestinal tract of most of the fish.

Further studies, considering growth and survival capacity at longer time periods, will clarify the effectiveness of LAB as biological control for undesirable bacteria during turbot culture.

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